IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Axel ULLRICH et al

Serial No.: 09/461,090 Art Unit No.: 1643

Filed: December 14, 1999

For: EGF RECEPTOR TRANSACTIVATION BY G-PROTEIN-COUPLED RECEPTORS REQUIRES METALLOPROTEINASE CLEAVAGE OF proHB-EGF

CLAIM TO PRIORITY UNDER 35 USC 119

Assistant Commissioner for Patents
Washington, D.C. 20231

April 4, 2000

Sir:

The benefit of the filing date of the following prior foreign application is hereby requested for the above-identified application, and the priority provided in 35 USC 119 is hereby claimed:

European Patent Application No. 99116056.5 filed August 16, 1999.

In support of this claim, the requisite certified copy of said original foreign application is filed herewith.

It is requested that the file of this application be marked to indicate that the applicants have complied with the requirements of 35 USC 119 and that the Patent and Trademark Office kindly acknowledge receipt of this document.

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Respectfully submitted,
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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

99116056.5

Der Präsident des Europäischen Patentamts;
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Sheet 2 of the certificate
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Growth-factor receptor activation by G-protein-mediated proteinase cleavage of precursor molecules

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Growth-Factor Receptor Activation by G-Protein-Mediated Proteinase Cleavage of Precursor Molecules

Growth-Factor Receptor Activation by G-Protein-Mediated Proteinase Cleavage of Precursor Molecules

Specification

The present invention relates to agents and methods for growth-factor receptor activation by modulating the activity of proteinases.

Crosstalk between different signalling systems allows the integration of a great diversity of stimuli that a cell receives under varying physiological situations. Transactivation of EGF receptor-dependent signalling pathways upon stimulation of G-protein-coupled receptors (GPCR) which are critical for the mitogenic activity of ligands such as LPA, endothelin, thrombin, bombesin and carbachol represents evidence for such an interconnected communication network. The mechanism of this cross-communication is not understood, but based on reported data it was proposed to be transmitted by intracellular elements[1-4].

We report here that activation of growth-factor receptors such as epidermal growth-factor receptor (EGFR) upon GPCR stimulation requires the receptor's extracellular domain. As key element of this mechanism we identify a membrane-spanning growth-factor ligand precursor, such as proHB-EGF, and a proteinase activity that is rapidly induced upon GPCR-ligand interaction. We show that inhibition of growth-factor precursor processing blocks GPCR-induced growth-factor receptor transactivation and downstream signals. As evidence for the pathopysiological significance of this mechanism we demonstrate inhibition of constitutive EGFR activity upon treatment of human PC-3 prostate carcinoma cells with the metalloproteinase inhibitor batimastat. Together, these results establish a new mechanistic concept for crosstalk among different signalling systems. Further, the results demonstrate the importance of proteinases as targets

for the treatment or prevention of diseases which are associated with pathological growth-factor receptor overexpression.

In a first aspect the invention relates to the use of modulators of G-protein mediated signal transduction for the manufacture of an agent which modulates growth-factor receptor activation. Preferably the activation of the growth-factor receptor is mediated by its extracellular domain and via an extracellular signal pathway. Thus the modulator may act on cells which are heterologous to the growth-factor receptor carrying target cells. The growth-factor receptor activation preferably occurs by tyrosine phosphorylation, by which an intracellular signal cascade is mediated. Examples of suitable growth-factor receptors are EGFR, and other members of the EGFR family such as HER-2, HER-3 or HER-4, but also other growth-factor receptors such as TNF receptor 1, TNF receptor 2, CD 30 and IL-6 receptor.

The modulator of the G-protein mediated signal transduction may act on one or several compounds of the signal transduction pathway. Particularly, the modulator may act on a G-protein, a G-protein coupled receptor and/or a proteinase which are key elements of the signal transduction pathway. Preferably the modulator acts on a proteinase.

The substrate which is subject to cleavage by the protease is preferably a growth-factor receptor ligand precursor. This precursor is preferably a membrane-associated molecule. In a particularly preferred example the growth-factor ligand precursor is proHB-EGF which is cleaved to HB-EGF and the growth-factor receptor is EGFR. Other preferred examples of growth-factor ligands which are cleaved from precursors are other members of the EGF family such as TGFα, amphiregulin, epiregulin, EGF, betacellulin, members of the heregulin/NDF family including isoforms thereof and TNFα.

The proteinase which is modulated is usually a membrane-associated proteinase, preferably a metalloproteinase such a zinc-dependent proteinase. The modulation of proteinase activity may comprise a stimulation or inhibition. Preferably the proteinase activity is inhibited which in turn results in an inhibition of growth-factor receptor activation.

The modulation of proteinase activity is preferably effected by adding an acitvator or inhibitor of proteinase activity to the system which in a particulary preferred embodiment directly modulates the proteinase activity. A preferred example for such a modulator for proteinase activity is the proteinase inhibitor batimastat. Further examples are marimastat (British Biotech), TAPI (Immunex) and TIMP-1, -2, -3 or -4, particularly TIMP-3[31].

The modulation of G-protein modulated signal transduction has great significance for diagnostic and clinical applications. For example, the modulation of G-protein mediated signal transduction is a target for the prevention or treatment of disorders associated with or accompanied by a disturbed e.g. pathologically enhanced growth-factor receptor acitvation. For example, the growth of human prostate cancer cells may be inhibited by treatment with batimastat. Further examples of growth-factor receptor associated disorders are asthma or infectious diseases, e.g. infections by bacteria such as Helicobacter.

Thus, the present invention provides a method for modulating growth-factor activation comprising contacting a cell or an organism which contains a growth-factor receptor capable of being activated with a modulator of G-protein mediated signal transduction. The contacting step may occur in vitro, e.g. in a cell culture or in vivo, e.g. in a subject in the need of medical treatment, preferably a human. The active agent is added in an amount sufficient to modulate growth-factor receptor activation, particularly in an amount sufficient to inhibit growth-factor receptor activation at least partially. Preferably the active agent is administered as a pharmaceutically

acceptable composition, which may contain suitable diluents, carriers and auxiliary agents. The composition may also contain further pharmaceutically active agents e.g. cytotoxic agents for the treatment of cancer. The dosage of the active agent depends on the specific compound being administered and the type and the severity of the disease to be treated. For batimastat, and other compounds e.g. a daily dosage of 1 to 200 mg/kg, particularly 10 to 100 mg/kg per day is suitable.

Still a further aspect of the present invention is a method for identifying and providing modulators of G-protein mediated signal transduction comprising contacting a cell which contains a growth-factor receptor capable of being activated with a test compound suspected to be a modulator of G-protein mediated signal tranduction and determining the degree of growth-factor receptor activation. This method is suitable as a high-throughput screening procedure for identifying novel compounds or classes of compounds which are capable of modulating G-protein signal transduction. Further, the present invention encompasses any novel modulator identified by the disclosed method.

Further, the present invention is described in detail by the following figures and examples:

Description of Figures

Figure 1 GPCR-induced EP-R transactivation redefines endogenous EGFR-mediated signalling to PDGFR-specific signals. Proteins were immunoblotted with *a*PY antibody (4G10).

a) Rat-1/EP-R cells were 3 minutes treated with ET-1 (200 nM), thrombin (2U/ml) and EGF (2ng/ml) or

b) preincubated with tyrphostins as indicated prior to thrombin stimulation and EP-R was selectively precipitated with mAb 108.1.

c) Different stable Rat-1 cell lines were untreated or

d) 1h preincubated with EGFR-E Ab ICR-3R (20μg/ml), stimulated for 3 minutes with GPCR agonists, EGF or PDGF-BB (25 ng/ml) as indicated and SHP-2 was precipitated.

e) Rat-1/EP-R were treated as in b) and SHC was immunoprecipitated.

Figure 2 Carbachol-induced intercellular transactivation of the EGF receptor. Stable Rat-1 cell lines either expressing M1R or HERc and control cells were mixed in 1:3 ratio. In

a) after stimulation with carbachol (1mM), HERc was precipitated and immunoblotted with αPY antibody.

b) Co-cultures of Rat-1/M1R and Rat-1/HERc cells were planted in different densities, preincubated with EGFR-E blocking Ab ICR-3R (20μg/ml) and HERc was precipitated following carbachol-stimulation.

Figure 3 GPCR-induced EGFR transactivation and adapter protein tyrosine phosphorylation is dependent on HB-EGF function. a), c), d) COS-7 and b) HEK 293 cells, transfected with the M1R or ET-R, respectively, untreated or CRM197 preincubated, were stimulated for 3 minutes with the GPCR agonists LPA (10 μM) or Carbachol (1 mM), EGF (2ng/ml) or 1 μM TPA (5 min) as indicated. Subsequently EGFR (a,b), SHC (c) or Gab 1(d) was immunoprecipitated and proteins were immunoblotted with αPY antibody (4G10).

Figure 4 GPCR-induced proteolytic processing of proHB-EGF and EGFR transactivation are critically dependent on metalloproteinase function.

a) COS-7 cells were co-transfected with either M1R or BombR (0.5 μg each) and VSV-proHB-EGF (0.7 μg) and stimulated

with carbachol (1 mM), bombesin (200 nM), TPA (1 μM) or EGF (2 ng/ml). ProHB-EGF was analysed with αHB-EGF Ab (upper part), cleaved VSV-HB-EGF was monitored by anti VSV immunoblotting (lower part).

b) COS-7 cells transfected as in a) were preincubated with batimastat (5 μM, 30 min), stimulated as indicated and anti-VSV immunoprecipitates were subjected to αHB-EGF immunoblotting.

c) Flow cytometric analyses of proHB-EGF in COS-7 cells treated for 10 minutes with LPA, TPA, EGF or batimastat preincubation following LPA stimulation.

d,e) COS-7 cells, transfected with the M1R, untreated or BB-94 preincubated, were stimulated as in Fig. 3a) and EGFR (d) or SHC (e) were immunoprecipitated. Proteins were immunoblotted with αPY antibody (4G10).

f) PC-3 cells were serum-starved for 36 hours, preincubated with batimastat and stimulated for 3 minutes with bombesin, TPA or EGF (7ng/ml) as indicated. EGFR was immunoprecipitated and immunoblotted with αPY antibody.

g) Unstarved PC-3 cells were treated for indicated times with DMSO or batimastat and EGFR tyrosine phosphorylation was monitored with αPY immunoblot.

Examples

1. Methods

Cloning and plasmids

The following plasmids have been described: pcDNA1-BombR and pcDNA3-M1R[1]. For stable expression of the M1R in Rat-1 cells the receptor was subcloned into pLXSN. pro-HB-EGF and the Endothelin receptor were amplified by PCR from a MCF-7 or Rat-1 cDNA library and subcloned into pcDNA3-VSV or pcDNA3, respectively.

Cells and transfections

Rat-1 cells and COS-7 cells were grown and infected or transfected, respectively, as described[1,2]. Rat-1HERc cells have been described elsewhere[1]. HEK 293 cells were grown in DMEM containing 10% FCS and transfections were carried out using the Ca-phosphate method. CRM197 (10 μg/ml,Sigma) or batimastat (BB-94), (5 μM, British Biotech) were added 20 minutes before the respective growth-factor. Tyrphostin AG1478 (250 nM, Calbiochem) and AG1295 (1 μM, Calbiochem) were added 15 minutes before stimulation.

Immunoprecipitation and Western blotting

The antibodies against human EGFR (108.1), SHP-2, Shc and Gab1 have been characterized [1,12,19,2]. Western blotting against the EP-R chimera was performed using rabbit polyclonal α-hPDGFRß antibody (Upstate Biotechnology). Cells were lysed and proteins were subsequently immunoprecipitated as described [1]. To precipitate the VSV-tagged HB-EGF a monoclonal VSV antibody (P5D4, Boehringer) in combination with Protein G-Sepharose was used, HB-EGF was detected with antibody C-18 (Santa-Cruz). Due to the small size of pro-HB-EGF and the processed form of HB-EGF we used the Tricine SDS-PAGE system established by Schlägger as described[30].

Flow cytometry analysis

COS-7 cells were seeded in 6 cm-dishes; 20h later cells were washed and cultured for a further 24h in serum-free medium until treatment with growth factors as indicated. After collection cells were incubated with goat αHB-EGF antibody (R&D Systems) for 30 minutes on ice. After washing with PBS, cells were incubated with FITC-conjugated rabbit anti-goat antibody (Sigma) for 20 minutes on ice. Cells were analysed with FACSCalibur (Becton Dickinson).

2. Results

Epidermal growth-factor receptor (EGFR) transactivation was identified as a critical element in mitogenic signalling [1,5,6] induced by G-protein-coupled receptors (GPCR), regulation of chloride channels[7], as well as modulation of potassium channel activity [8]. Since the process was found to be very rapid[1,7,9], and GPCR-induced release of EGFR ligands into the cell culture medium could not be detected[5,8], EGFR transactivation has been generally assumed to be exclusively mediated via intracellular signals[3,4].

Surprisingly, however, even though PDGF receptors are not transactivated upon treatment of Rat-1 cells with GPCR ligands[2], this was the case for a chimera EP-R consisting of an EGFR extracellular and the platelet-derived growth-factor receptor (PDGFR) transmembrane and cytoplasmic signalling domain[10] (Fig. 1a). This receptor chimera immunoprecipitates with monoclonal antibody 108.1 which recognized the extracellular portion of human but not rat EGFR. Treatment of Rat-1/EP-R cells with the PDGFR inhibitor AG1295[11], but not with the EGFR kinase antagonist AG1478[1], blocked thrombin-induced tyrosine phosphorylation of the chimeric receptor (Fig. 1b), which clearly demonstrated a critical function of the EGFR extracellular domain for GPCR-mediated transactivation. As shown in Fig. 1c, this EP-R transactivation results in a PDGF-characteristic downstream signal, since the SH2 domain-cointaining phosphatase 2 (SHP-2), a preferred mediator of PDGFR signalling[12], was tyrosine phosphorylated upon endothelin (ET-1) and thrombin stimulation of Rat-1/EP-R cells, while exposure to the same ligands did not induce SHP-2 tyrosine phosphoryation in Rat-1 cells overexpressing the PDGFR or control cells. Pretreatment of Rat-1/EP-R cells with monoclonal antibody ICR-3R[13] that blocks ligand binding to the human EGFR resulted in complete inhibition of ET-1 and EGF-

induced SHP-2 tyrosine phosphorylation, whereas the PDGF-mediated response was not affected (Fig. 1d), confirming that GPCR-induced transactivation of the EP-R chimera depends on the extracellular EGFR domain. In contrast to the results obtained for SHP-2 (Fig. 1c), tyrosine phosphorylation of the adaptor protein SHC following thrombin stimulation was completely blocked by pretreatment of Rat-1/EP-R cells with AG1478, but remained unaffected by preincubation with the PDGFR antagonist AG1295 (Fig. 1e). This confirms that thrombin transactivates endogenuos rat EGFR in Rat-1/EP-R cells resulting in SHC tyrosine phosphorylation, whereas activation of the EP-R chimera redefines thrombin stimulation to generate a PDGFR-characteristic SHP-2 signal.

To address the question whether the extracellular signal which activates the EP-R chimera acts via an autocrine or paracrine mode, we performed a co-culture experiment with Rat-1 cells either stably overexpressing the M1 muscarinic acetylcholine receptor (M1R) or the human EGFR (HERc) at a ratio of one to one. Stimulation of the Rat-1/M1R + Rat-1/HERc co-culture with the M1R agonist carbachol prior to immunoprecipitation with human EGFR-specific antibody 108.1, rapidly induced tyrosine phosphorylation of HERc (Fig. 2a). Since neither of the control cells responded to carbachol, this result clearly demonstrated the possibility of transactivation between two cells. To investigate the influence of cell density on this paracrine process, HERc was immunoprecipitated from subconfluent versus confluent co-cultures of Rat-1/M1R and Rat-1/HERc cells following stimulation with carbachol. As shown in Fig. 2b, EGFR tyrosine phosphorylation in response to M1R agonist only occurred in confluent co-cultures and was completely inhibited by preincubation with ICR-3R antibody. This further demonstrated the requirement of the EGFR ligand binding function for intercellular signal transmission and the necessity of close cell-cell contact. Together, these results

lead us to conclude that EGF-like ligands, synthesized as transmembrane precursors and converted to the mature form by proteolytic cleavage[14], may be involved in GPCR-mediated transactivation. The discrepancy between previous results obtained from medium-transfer experiments[5,8] and our finding of density-dependent intercellular crosstalk might be due to a scenario in which upon proteolytic processing EGF-like ligands remain with the heparin sulfate proteoglycan matrix prior to interaction with their high-affinity receptors as shown for fibroblast growth-factors[15].

Ectodomain shedding has been shown to be induced by stimuli such as activators of heterotrimeric G-proteins, AlF_4^- and GTPγS[16], as well as the PKC activator tetradecanoyl-phorbol-13-acetate (TPA) and the Ca^{2+}-ionophore ionomycin[17,18]. The latter, which induces HB-EGF release in prostate epithelial cells[18], has recently been shown to be a potent activator of EGFR transactivation in PC12 cells[19], and TPA has been reported to induce EGFR tyrosine phosphorylation in HEK 293 cells[8]. HB-EGF, a member of the EGF family, has the ability to bind to cell surface heparan sulfate proteoglycans[20], which prevents the immediate releae of the growth-factor and increases the local growth factor concentration in the cellular microenvironment. Based on these properties the proHB-EGF precursor matched our proposed requirement for GPCR-induced EGFR transactivation. Besides its function as a growth-factor precursor, proHB-EGF serves as a high-affinity receptor for diphteria toxin (DT)[21]. CRM197, a non toxic mutant of DT, was shown to inhibit strongly and specifically the mitogenic activity of HB-EGF[22]. Therefore, we tested the influence of CRM197 on GPCR-mediated EGFR transactivation. We found that CRM197 pretreatment completely inhibits tyrosine phosphorylation of the EGFR induced by the GPCR agonists lysophosphatidic acid (LPA) or carbachol as well as TPA in COS-7 cells (Fig. 3a). Inhibition was also observed for ET-1 or TPA-stimulated HEK 293 cells

transiently transfected with the endothelin receptor (Fig. 3b). In contrast, EGF-induced receptor tyrosine phosphorylation was unaltered demonstrating CRM197 specificity. Furthermore, complete abrogation of LPA- and carbachol-induced receptor tyrosine phosphorylation suggested that HB-EGF is the only growth-factor mediating EGFR transactivation in the cell lines presented here.

Tyrosine phosphorylation of the adaptor protein SHC is considered to be a critical step in the coupling of GPCR activation to Ras-dependent signalling pathways[23]. In order to investigate the role of HB-EGF in this process, we examined the effect of the diphteria toxin mutant CRM197 on GPCR ligand and TPA-mediated SHC tyrosine phosphorylation. As shown in Fig. 3c, in COS-7 cells, LPA-, carbachol-and TPA-induced SHC tyrosine phosphorylation was dramatically reduced by CRM197 pretreatment, while the EGF-mediated response was not affected. The same inhibitory effect of CRM197 was observed in HEK 293 cells (data not shown). Similarly, in COS-7 cells, tyrosine phosphorylation of the multidocking protein Gab1 in response to LPA or thrombin was not detected in the presence of CRM197 (Fig. 3d) confirming its signalling position downstream of the EGFR[2].

Next, in order to examine whether proHB-EGF is proteilytically processed upon stimulation of GPCRs, we transfected plasmids containing VSV-tagged proHB-EGF in COS-7 cells together with the M1R or the bombesin receptor (BombR) and stimulated with respective ligands for different times. TPA, a potent inducer of proHB-EGF processing, or EGF were added as positive and negative controls, respectively. Figure 4a shows that as previously described proHB-EGF is expressed in form of heterogenous translation products of 20 to 30 KDa[17], which can be detected with antibodies against the C-terminus of the precursor (upper panel) or the VSV-tag (lower

panel). Stimulation with carbachol or bombesin led to a rapid breakdown of the membrane-anchored growth-factor precursor and proteolytic cleavage was concommitant with the appearance of the 9 KDa VSV-tagged HB-EGF fragment containing the transmembrane anchor. Interestingly, under these conditions the GPCR signal induced proteolytic proHB-EGF proccessing as fast and potently as TPA. As for TPA[17], GPCR-induced conversion of proHB-EGF is an extremely rapid process that generates mature HB-EGF. In contrast to GPCR-induced tyrosine phosphorylation of endogenous EGFR which is fast and transient[1,7,9], overexpression of the protease substrate VSV-proHB-EGF led to a rapid but more sustained ectodomain cleavage of proHB-EGF.

Since zinc-dependent metalloproteinases have been implicated in pro-HB-EGF shedding by TPA[24], we analysed carbachol-induced processing in the presence of batimastat (BB-94)[25], a protease inhibitor which has recently been shown to block proteolytic maturation of human amphiregulin[26]. As shown in Fig. 4b, BB-94 treatment significantly reduced HB-EGF processing in response to carbachol supporting our conclusion that metalloproteinases are critical elements in GPCR-induced HB-EGF generation and EGFR activation.

To confirm GPCR-induced proHB-EGF processing, we used an ectodomain-specific antibody and flow cytometry upon treatment of non-transfected COS-7 cells with LPA, TPA or EGF. Within 10 minutes after addition of LPA and TPA, the content of cell surface proHB-EGF was reduced while EGF stimulation showed no effect (Fig. 4c). In contrast to the experiments with transfected cells shown in Fig. 4a and b, activation of endogenous LPA receptors was not as potent as TPA to induce proteolytic cleavage of proHB-EGF.

Nonetheless, consistent with Fig. 4b, the modest LPA-induced effect was completely inhibited by batimastat.

Our results demonstrate that metalloproteinase-dependent cleavage of proHB-EGF is rapidly induced upon activation of GPCRs and consequently suggest a critical and general role of this process in EGFR transactivation. We therefore investigated the effect of the metalloproteinase inhibitor batimastat in GPCR- as well as TPA-induced EGFR transactivation. In COS-7 cells, BB-94 pretreatment completely abrogated LPA- and carbachol-induced tyrosine phosphorylation of the EGFR, as well as TPA-mediated receptor activation (Fig. 4d). Since TPA- but not GPCR-mediated EGFR tyrosine phosphorylation is sensitive to PKC inhibition in COS-7 cells (data not shown), it appears that at least two distinct metalloproteinase-dependent transactivation pathways exist. Analogous results were obtained for ET-1-induced transactivation in HEK 293 cells and bradykinin-stimulated EGFR tyrosine phosphorylation in PC12 cells (data not shown). Finally, the general implication of proteolytic processing in EGFR transactivation and downstream signal transmission is demonstrated by the complete abrogation of GPCR- and TPA-induced SHC tyrosine phosphorylation by batimastat (Fig. 4e).

Because of the well established role of EGFR family members in the pathogenesis of a variety of cancers and the physiological abundance of GPCR ligands such as LPA, we addressed the pathophysiological significance of transactivation with the human prostate cancer cell line PC-3 which has been reported to utilize EGFR-dependent pathways for growth promotion and is also responsive to the GPCR ligand bombesin[27,28]. Figure 4f shows that in PC-3 cells that were starved for 36 hours, bombesin, TPA and EGF induce tyrosine phosphorylation of the EGFR which is completely blocked by

batimastat-treatment. Moreover, even high constitutive phosphotyrosine content of the EGFR in unstarved PC-3 cells is reduced by long-term treatment with BB-94 (Fig. 4g). All in all, our results allow the conclusion that metalloproteinase-mediated precursor cleavage represents a direct link between BombR activation, constitutive tyrosine phosphorylation of the EGFR and proliferation of human prostate cancer cells. Recently, ADAM9, a member of the metalloproteinase-disintagrin family has been reported to process proHB-EGF upon TPA treatment of Vero-H cells[24]. We were unable, however, to block EGFR transactivation with dominant-negative ADAM9 mutants in COS-7 and HEK 293 cells (data not shown) leaving the identity of the precursor processing protease unresolved.

Our findings identify the ubiquitously expressed HB-EGF precursor and a metalloproteinase activity as critical pathway elements between GPCR signals and activation of the EGFR and extend our understanding of the mechanisms that underly the multiple biological processes known to be regulated by heterotrimeric G-proteins. Based on our current state of understanding, GPCR-induced EGFR signal transactivation represents a new paradigm because it entails three different transmembrane signal transmission events: First, a ligand activates heterotrimeric G-proteins by interaction with a GPCR which results in an intracellular signal that induces the extracellular activity of a transmembrane metalloproteinase. This then results in extracellular processing of a transmembrane growth-factor precursor and release of the mature factor which, directly or via the proteoglycan matrix, interacts with the ectodomain of the EGFR leading to intracellular autophosphorylation and signal generation. Our previous findings indicate that this pathway may be utilized by a variety of GPCRs in diverse cell types and that the preferred transactivation target is the EGFR and its relatives[1-4]. The

demonstration of the pathophysiological relevance of this novel mechanism in prostrate cancer cells leads us to propose that EGFR transactivation via G-protein-mediated proteolytic growth precursor processing represents a general mechanism with broad significance. Moreover, since a great variety of bioactive polypeptides as diverse as TNF-*a*, FAS-ligand or L-selectin are processing products of transmembrane precursors[29] that have been connected to pathophysiological disorders, our findings shed new light on the importance of membrane-associated proteinases as targets for disease intervention strategies.

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Claims

1. Use of modulators of G-protein mediated signal transduction for the manufacture of an agent which modulates growth-factor receptor activation.

2. The use of claim 1, wherein the activation of the growth-factor receptor is mediated by its extracellular domain.

3. The use of claim 1 or 2, wherein the growth-factor receptor is activated by tyrosine phosphorylation.

4. The use of any one of the previous claims, wherein said growth-factor receptor is EGFR.

5. The use of any one of the previous claims wherein the modulator acts on a G-protein, a G-protein coupled receptor and/or a proteinase.

6. The use of claim 5, wherein the modulator acts on a proteinase.

7. The use of claim 6, wherein said modulator acts on said proteinase by directly stimulating or inhibiting the proteinase activity.

8. The use of any one of claims 6 or 7, wherein said proteinase cleaves a growth-factor ligand precursor.

9. The use of claim 8, wherein said precursor is a membrane-associated molecule.

10. The use of claim 8 or 9, wherein said growth factor ligand precursor is proHB-EGF and said growth-factor receptor is EGFR.

11. The use of any one of claims 6-10, wherein said proteinase is a membrane-associated proteinase.

12. The use of any one of the claims 6-11, wherein said proteinase is a metalloproteinase.

13. The use of claim 12, wherein said metalloproteinase is a zinc-dependent proteinase.

14. The use of any one of claims 6 to 13, wherein said proteinase activity is inhibited by batimastat.

15. The use of any one of the previous claims for the prevention or treatment of disorders associated with or accompanied by a disturbed, e.g. pathologically enhanced growth-factor receptor activation.

16. The use of claim 15 for the treatment of cancer, infectious diseases or asthma.

17. A method for modulating growth-factor activation comprising contacting a cell or an organism which contains a growth-factor receptor capable of being activated with a modulator of G-protein mediated signal transduction.

18. The method of claim 17, wherein said modulator is administered as a pharmaceutically acceptable composition.

19. A method for identifying and providing modulators of G-protein mediated signal transduction comprising contacting a cell which contains a growth-factor receptor capable of being activated with a test compound suspected to be a modulator of G-protein mediated

signal transduction and determining the degree of growth-factor receptor activation.

Abstract

The present invention relates to agents and methods for growth-factor receptor activation by modulating the G-protein mediated signal transduction pathway.

Fig. 1a

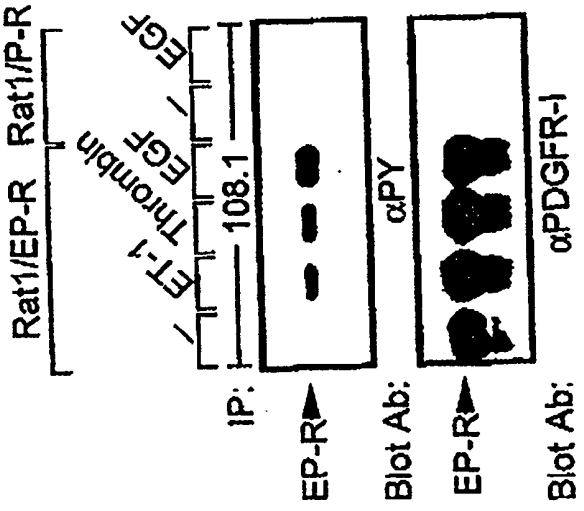


Fig. 1a

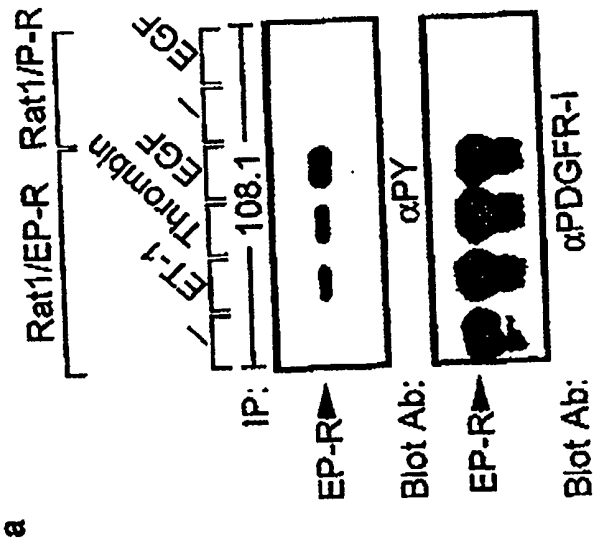


Fig. 1b

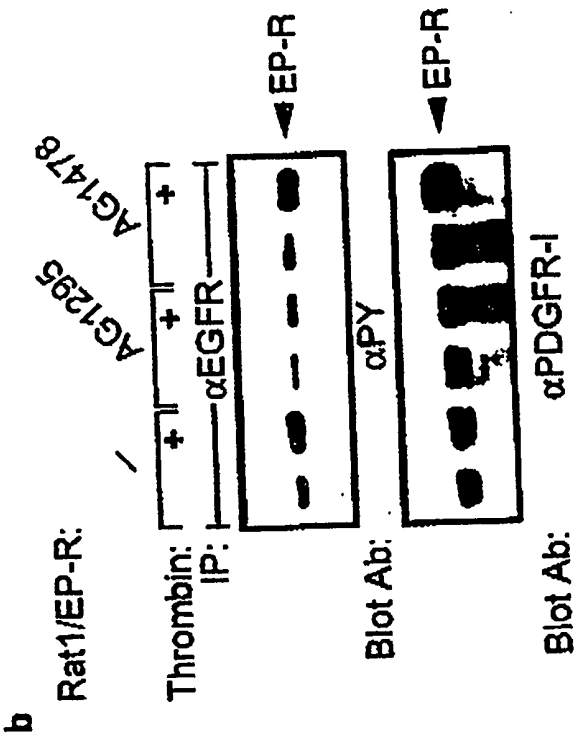

b
Rat1/EP-R:
Thrombin:
IP:
αEGFR
AG1295
AG1478
Blot Ab:
αPY
EP-R
Blot Ab:
αPDGFR-I
EP-R

Fig. 1b

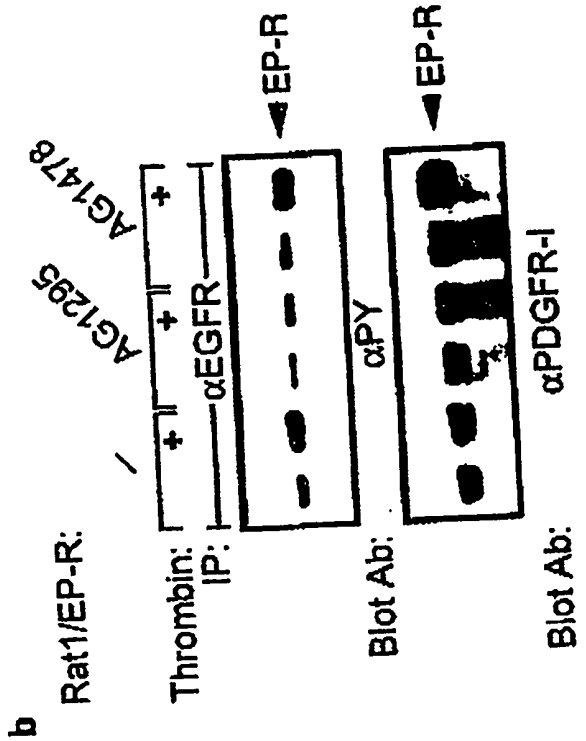


Fig. 1c

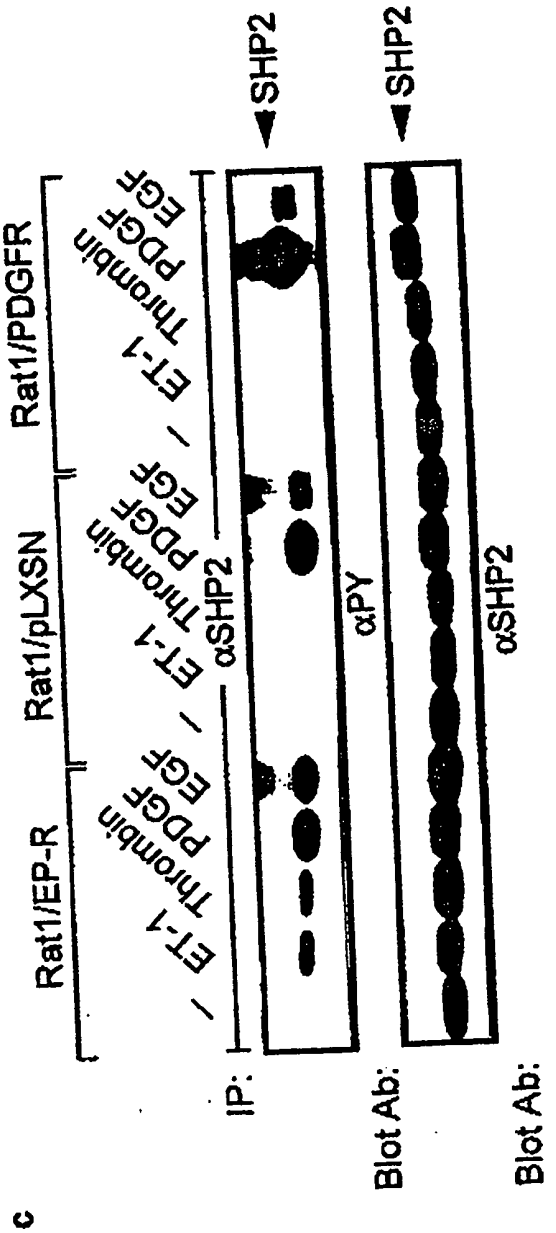


Fig. 1d

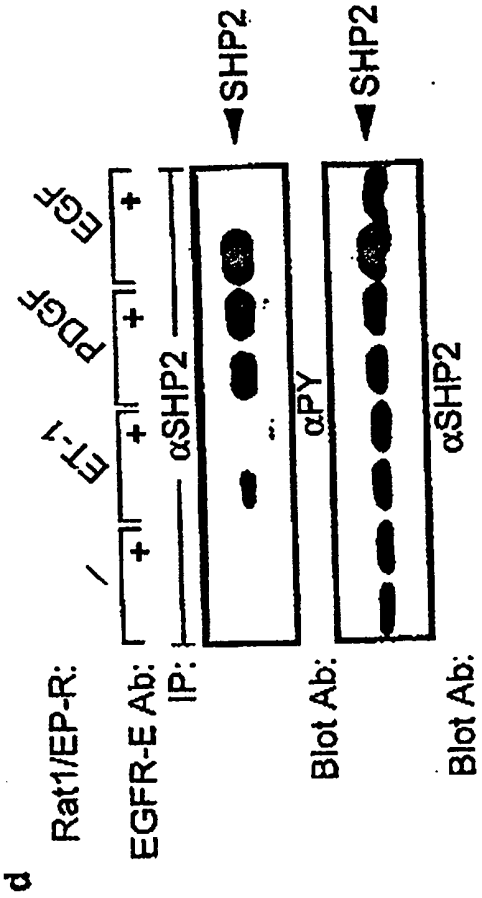


Fig. 1e

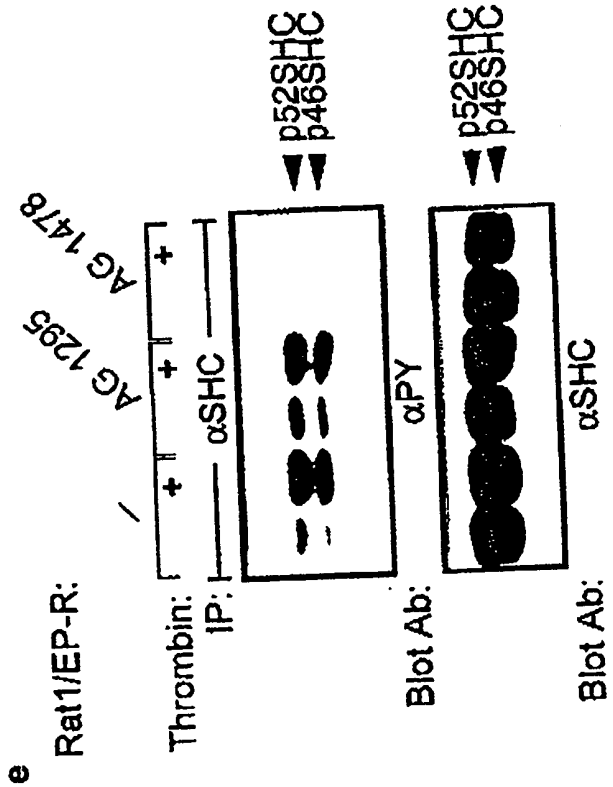


Fig. 2a

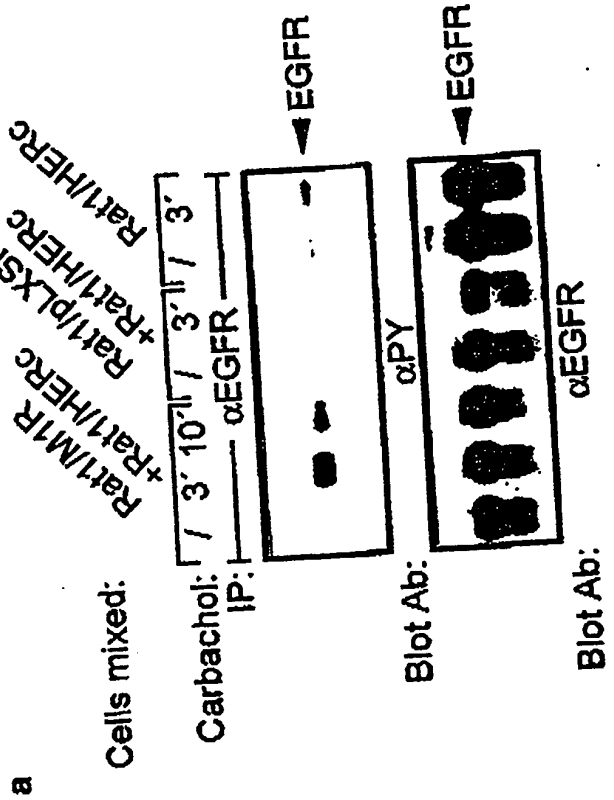


Fig. 2b

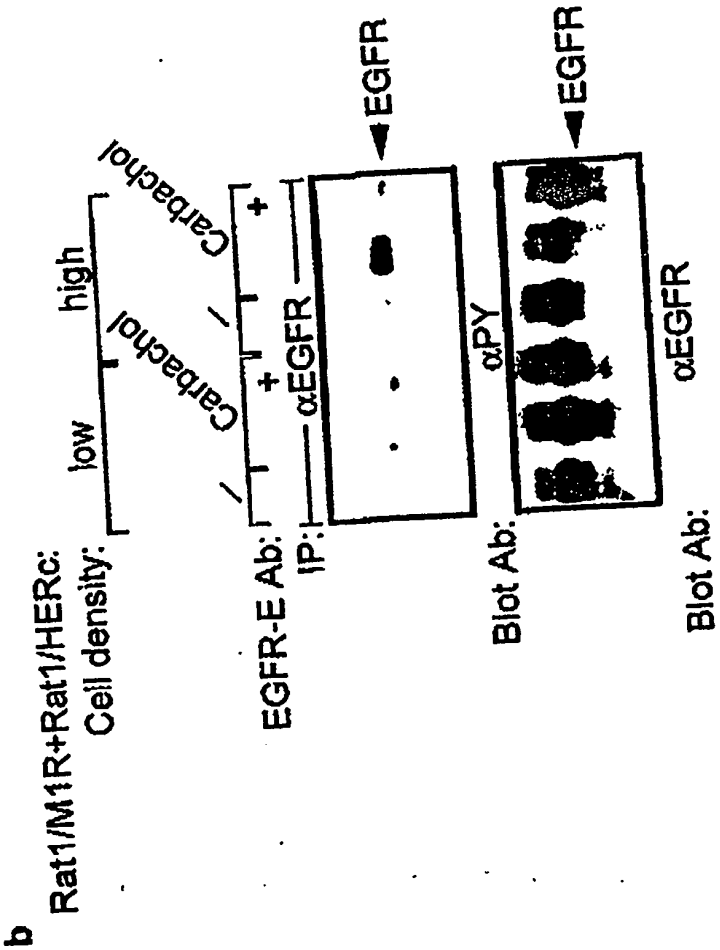
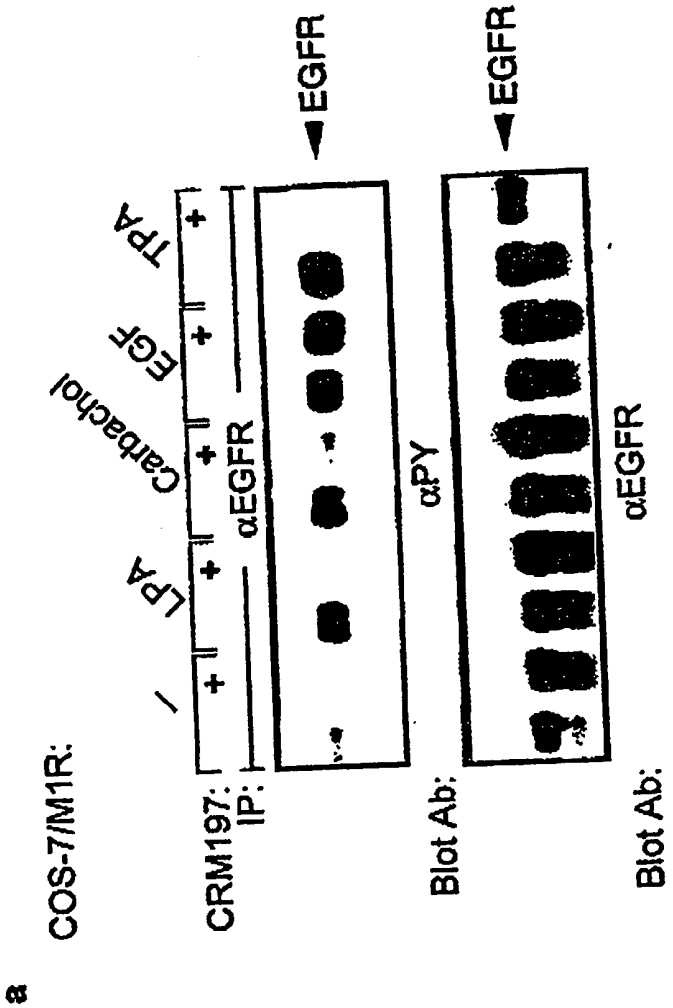


Fig. 3a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CRM197:
IP:
LPA
Carbachol
EGF
TPA
αEGFR
αPY
αEGFR
EGFR
EGFR
Blot Ab:
Blot Ab:

a

Fig. 3b

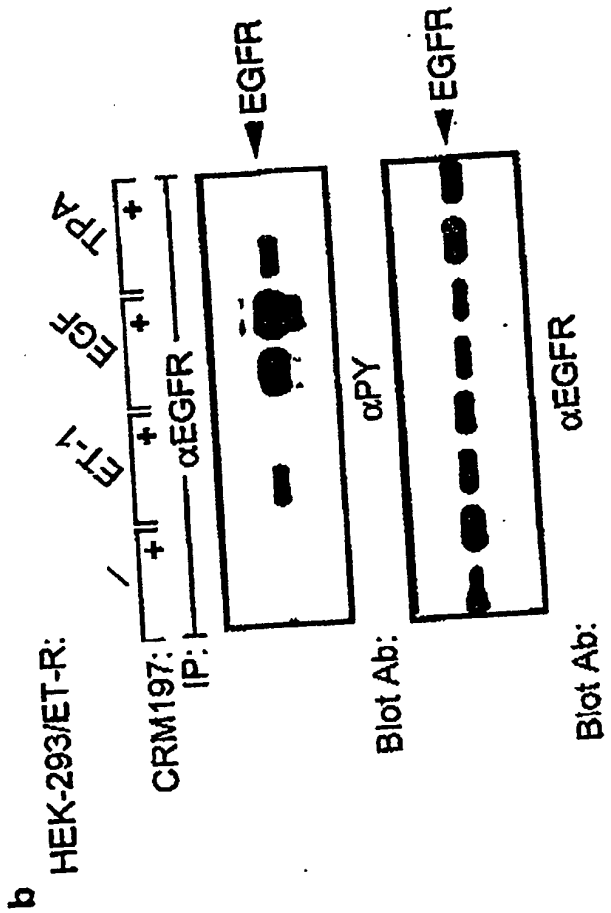
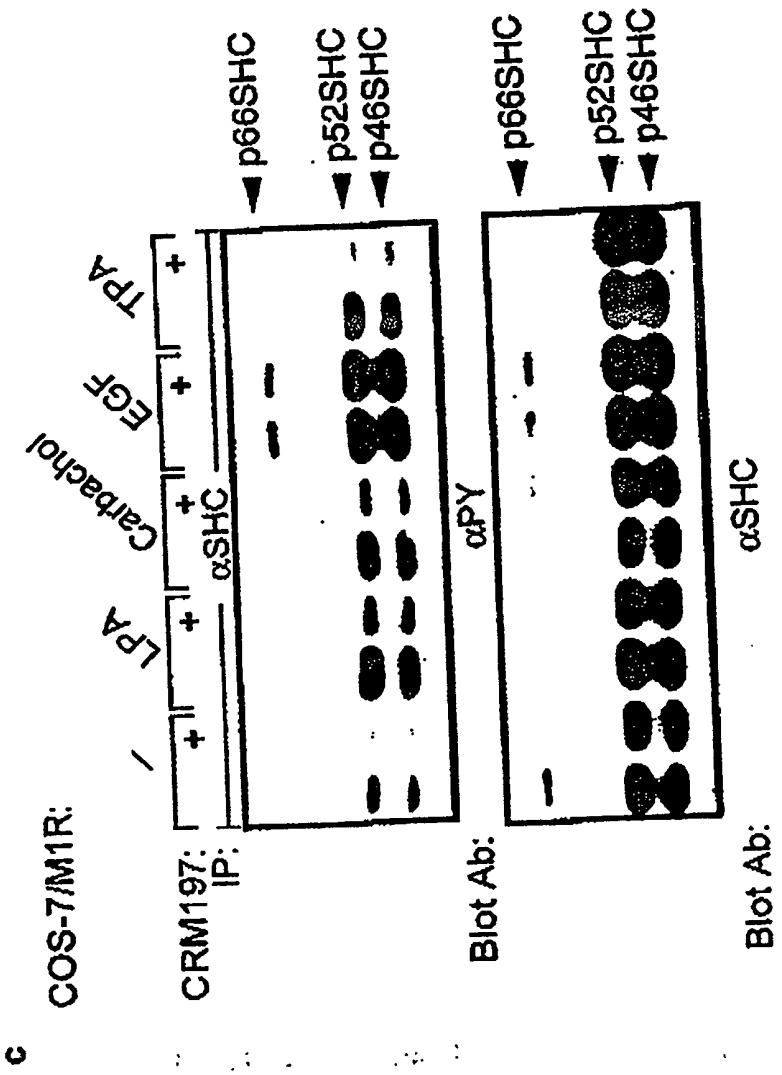


Fig. 3c


c
COS-7/M1R:
CRM197:
IP:
Blot Ab:
Blot Ab:
αSHC
αPY
αSHC
LPA
Carbachol
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TPA
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p46SHC
p66SHC
p52SHC
p46SHC

Fig. 3d

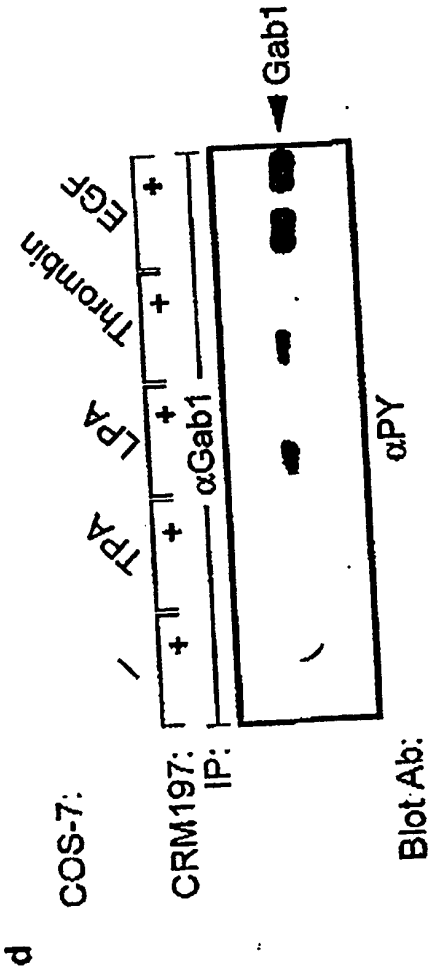


Fig. 4a

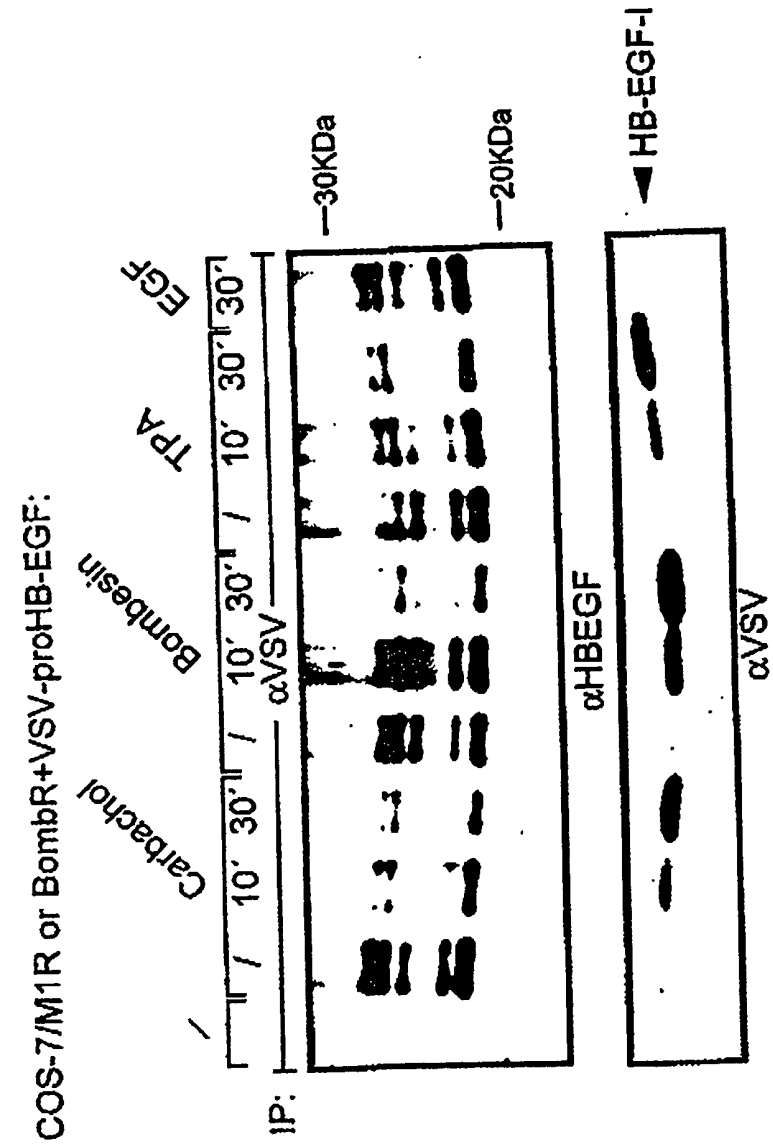
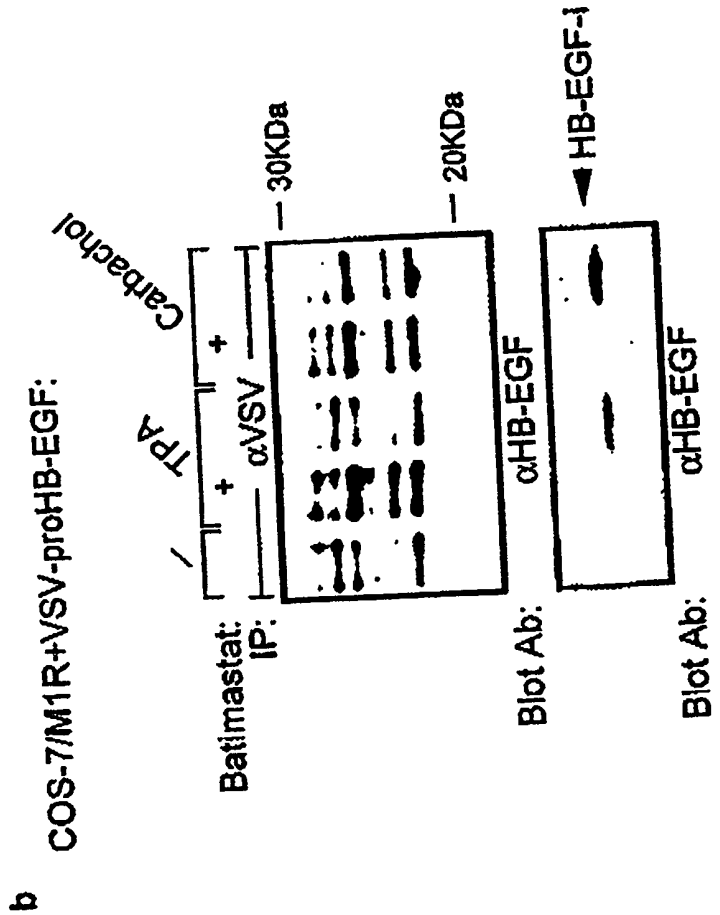

a
COS-7/M1R or BombR+VSV-proHB-EGF:
IP:
αVSV
Carbachol
Bombesin
TPA
EGF
10' 30' / 10' 30' / 10' 30' / 10' 30'
—30KDa
—20KDa
αHBEGF
αVSV
HB-EGF-I

Fig. 4b



Unstimulated
EGF
control
Relative cell number
Fluorescence Intensity
Unstimulated
Batimastat + LPA
control
Relative cell number
Fluorescence Intensity
Unstimulated
LPA
control
Relative cell number
Fluorescence Intensity
Unstimulated
TPA
control
Relative cell number
Fluorescence Intensity

Fig. 4d

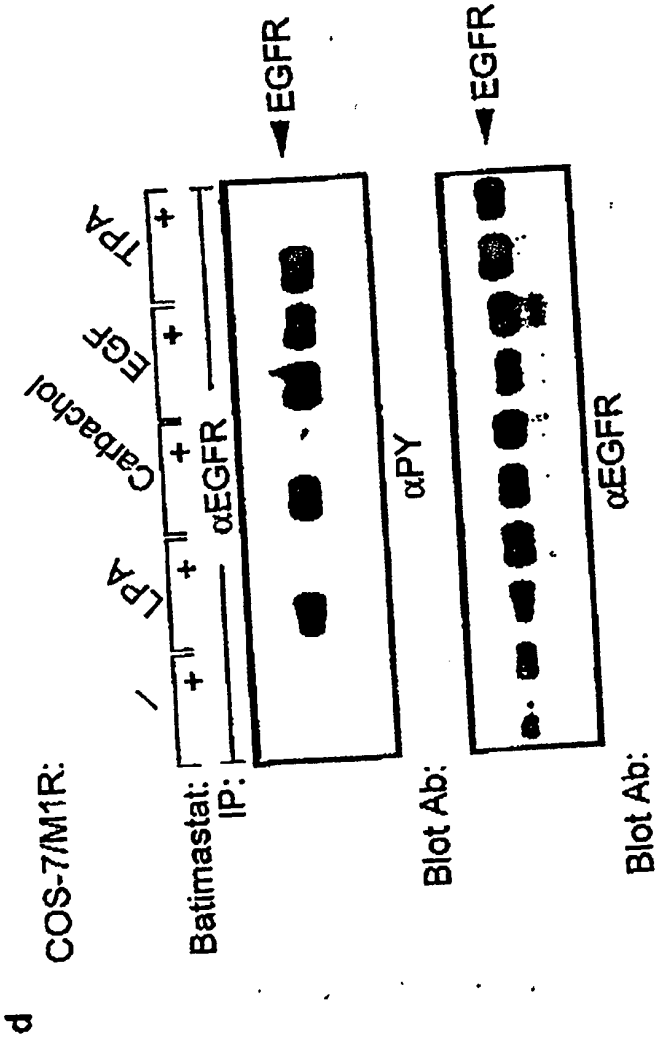


Fig. 4e

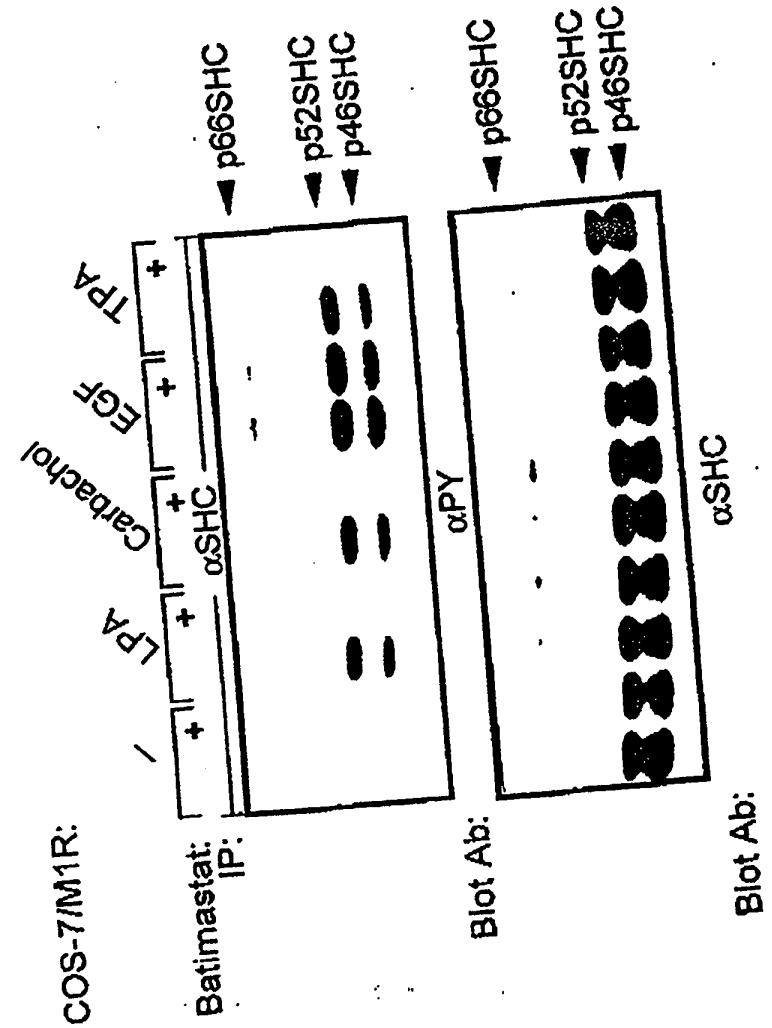


Fig. 4f

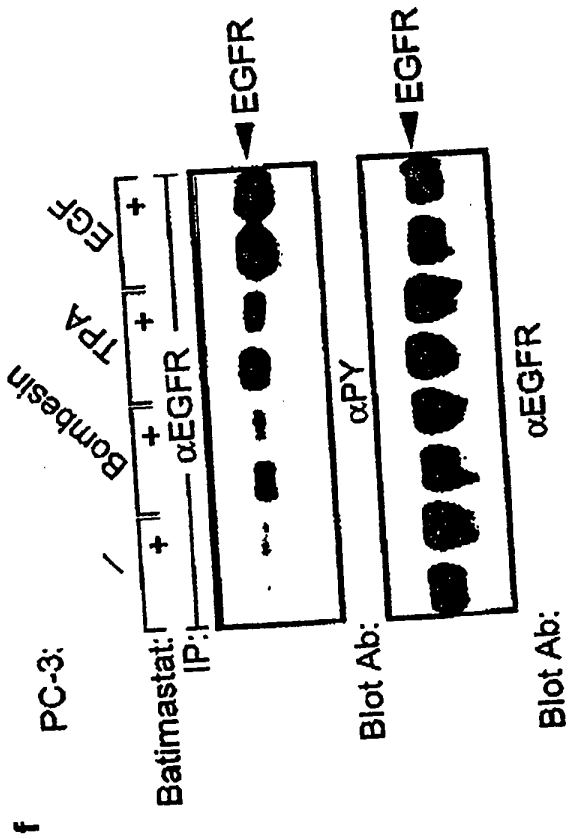


Fig. 4g